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# 5-Lipoxygenase Inhibition of the Fructus of *Foeniculum vulgare* and Its Constituents

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## Abstract

The fruits of *Foeniculum vulgare* (Foeniculi Fructus) have been widely used in Chinese medicine as an antiemetic, ameliorating stomach ailments and as an analgesic. In order to establish its potential for antiallergic use, inhibitory actions of the fruit on 5-lipoxygenase (5-LOX) and β-hexosaminidase release were evaluated. The 70% ethanol extract of this plant material (FR) considerably inhibited 5-LOX-catalyzed leukotriene production from A23187-induced rat basophilic leukemia (RBL)-1 cells. The $IC_{50}$ was 3.2 μg/ml. From this extract, 12 major compounds including sabinene, fenchone, γ-terpinene, α-pinene, limonene, *p*-anisylacetone, *p*-anisylaldehyde, estragole (4-allylanisole), *trans*-anethole, scopoletin, bergapten and umbelliferone were isolated. And it was found that several terpene derivatives including γ-terpinene and fenchone as well as phenylpropanoid, *trans*-anethole, showed considerable inhibitory action of 5-LOX. In particular, the $IC_{50}$ of *trans*-anethole was 51.6 μM. In contrast, FR and the isolated compounds did not show considerable inhibitory activity on the degranulation reaction of β-hexosaminidase release from antigen-treated RBL-2H3 cells. Against arachidonic acid-induced ear edema in mice, FR and *trans*-anethole showed significant inhibition by oral administration at doses of 100-400 mg/kg. In conclusion, FR and several major constituents are 5-LOX inhibitors and they may have potential for treating 5-LOX-related disorders.

**Key Words:** *Foeniculum vulgare*, *trans*-anethole, 5-lipoxygenase, Ear edema, Allergy

## INTRODUCTION

Leukotrienes (LTs) are mediators of inflammation and allergy. LTs, especially cysteinyl-LTs, are known to be involved in several allergic disorders including bronchial asthma and atopic dermatitis (Rubin and Mollison, 2007). Arachidonic acid (AA) released by phospholipase $A_2$ from membrane lipids is converted to LTs by 5-lipoxygenase (5-LOX). Thus, 5-LOX inhibitors have a potential to inhibit inflammatory/allergic response. In this regard, many synthetic small molecules and natural products are evaluated for their capacity to inhibit 5-LOX.

The fructus of *Foeniculum vulgare* (Foeniculi Fructus) is a well known Chinese traditional medicine. This plant has been widely used as an antiemetics, ameliorating stomach conditions, and as an analgesic (Him *et al.*, 2008). To present, many compounds have been isolated from this plant material. They include essential oils including *trans*-anethole, limonene, fenchone and cymene, fatty acids and coumarins such as scopoletin and bergapten (Ozcan and Chalchat, 2010). Previously, the fruits of *F. vulgare* extract were found to possess anti-inflammatory and analgesic activities (Choi and Hwang, 2004). The extract of the same plant material also showed anti-bacterial and anti-fungal activities (Cetin *et al.*, 2010; Pai *et al.*, 2010). As the constituents, essential oils such as fenchone and anethole showed antimicrobial and insecticidal activities (Kwon *et al.*, 2002; Cetin *et al.*, 2010). *Trans*-anethole and limonene also inhibit nitric oxide (NO) production from RAW 264.7 macrophages (Conforti *et al.*, 2010). In particular, essential oil fractions showed 5-LOX inhibitory activity (Miguel *et al.*, 2010), but the active principles were not identified. In this study, 5-LOX inhibitory activity of *F. vulgare* and its major constituents were examined in order to clearly establish the pharmacological action of *F. vulgare* and its major constituents as well as establish the potential of anti-allergic use.

## MATERIALS AND METHODS

### Chemicals

A23187 was obtained from Biomol (Plymouth Meeting,

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PA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), *p*-nitrophenyl-N-acetyl-β-D-glucosaminide, nordihydroguaiaretic acid (NDGA), quercetin, anti-dinitrophenol (DNP) mouse IgE, siraganian buffer, DNP-BSA, quercetin and arachidonic acid (AA) were purchased from Sigma Chem. (St. Louis, MO). DMEM and other cell culture reagents including FBS were products of Gibco BRL (Grand Island, NY). A protein assay kit was purchased from Bio-Rad (Hercules, CA).

## Animals

Male ICR mice (5 weeks old, specific pathogen-free) were obtained from Orient-Bio Co. (Korea). Animals were fed with standard lab. chow and water is freely available. The animals were maintained in the animal facility (KNU) at 20-22°C under 40-60% relative humidity and a 12 h/12 h (light/dark) cycle for at least 7 days prior to the experiment. The experimental design using the animals was approved by the local committee for animal experimentation, KNU (KIACUC-09-0029). The animals were handled according to the guideline described in the KFDA Guide for the Care and Use of Laboratory Animals throughout the experiments.

## Preparation of the extracts and isolation of the constituents

The fruits of *F. vulgare* cultivated in Neimenggu were provided from Prof. Jae-Hyun Lee, College of Oriental Medicine, Dongguk University at Gyeongju, Korea. Air-dried and chopped plant materials (1.0 kg) were extracted with hot 70% ethanol and hot distilled water for 3 h, respectively, to provide both extracts for the pharmacological activity test. For isolation of the constituents, plant materials (5.0 kg) were extracted with hot methanol for 3 h. Evaporation of the solvent yielded crude extracts, which were suspended in distilled water. The resulting solution was consecutively partitioned with hexane, methylene chloride, ethyl acetate and *n*-butanol to give hexane (221.4 g), methylene chloride (6.1 g), ethyl acetate (5.8 g), *n*-butanol (26.5 g). The hexane and methylene chloride fractions were subjected to column chromatographic separation. Coumarins (scopoletin, bergapten and umbelliferone) were isolated from the methylene chloride fraction as previously reported (Abdel-Fattah *et al.*, 2003). Other compounds including monoterpenes (sabinene, fenchone, γ-terpinene, α-pinene, limonene), phenylpropanoids (estragole and *trans*-anethole) and aromatics (*p*-anisylacetone and *p*-anisylaldehyde) (Fig. 1A) were obtained from the hexane fraction by isolation procedures according to the previously published procedure (Akgul, 1986). The spectral analysis of hexane fraction was performed with GC using HP-5MS capillary column (60.0 m× 250 μm×0.25 μm), and the spectrum was shown in Fig. 1B. The content of *trans*-anethole in hexane fraction was 23.3% (w/w). The purity of above isolated compounds were scopoletin 99.0%, bergapten 99.4%, umbelliferone 98.0%, sabinene 99.1%, fenchone 99.9%, γ-terpinene 95.0%, α-pinene 98.0%, limonene 97.2%, estragole 97.0%, *trans*-anethole 99.0%, *p*-anisylacetone 98.4%, *p*-anisylaldehyde 98.0%. The spectral data of the most active component, *trans*-anethole are as follows: $^1$H-NMR (200 MHz, $CDCl_3$) δ: 1.87 (3H, dd, *J*=6.7, 1.5 Hz, H-3'), 3.79 (3H, s, H-7), 6.10 (1H, dq, *J*=15.8, 6.7 Hz, H-2'), 6.35 (1H, dq, *J*=15.8, 1.5 Hz, H-1'), 6.84 (2H, dt, *J*=8.7, 3.0, 1.9 Hz, H-2, H-6), 7.25 (2H, dt, *J*=8.7, 3.0, 1.9 Hz, H-3, H-5); $^{13}$C-NMR (50 MHz, $CDCl_3$) δ: 18.2 (C-3'), 55.2 (C-7), 123.5 (C-2'), 130.4 (C-1'), 113.8 (C-2, C-6), 126.8 (C-3, C-5), 158.6 (C-1), 130.8 (C-4); EI-GC/MS *m/z* 148 [M]+.

## Rat basophilic leukemia-1 (RBL-1) cell culture and measurement of leukotriene (LT)

To evaluate the 5-LOX inhibitory activity, RBL-1 cells purchased from the American Type Culture Collection (ATCC, Rockville, VA) were cultured in RPMI 1640 with 10% FBS, 2 mM glutamine and 1% antibiotics under 5% $CO_2$ at 37°C. The 5-LOX activation was carried out by treatment of A-23187 (3 μM) for 15 min according to the previously described (Tries *et al.*, 2002). The test compounds were dissolved in DMSO and they were added to the cells simultaneously with A-23187. The cell viability was assessed using an MTT assay as previously described (Mosmann, 1983). The media was then collected and the concentration of the 5-LOX product, cysteinyl leukotrienes ($LTC_4/D_4/E_4$), was measured using an ELISA kit (Cayman Chem.) as recommended by the manufacturer.

## RBL-2H3 cell culture and antigen-induced degranulation of β-hexosaminidase

RBL-2H3 cells (ATCC) were cultured in 24-well plates ($2\times10^5$ cells/well) using DMEM with 10% FCS. According to

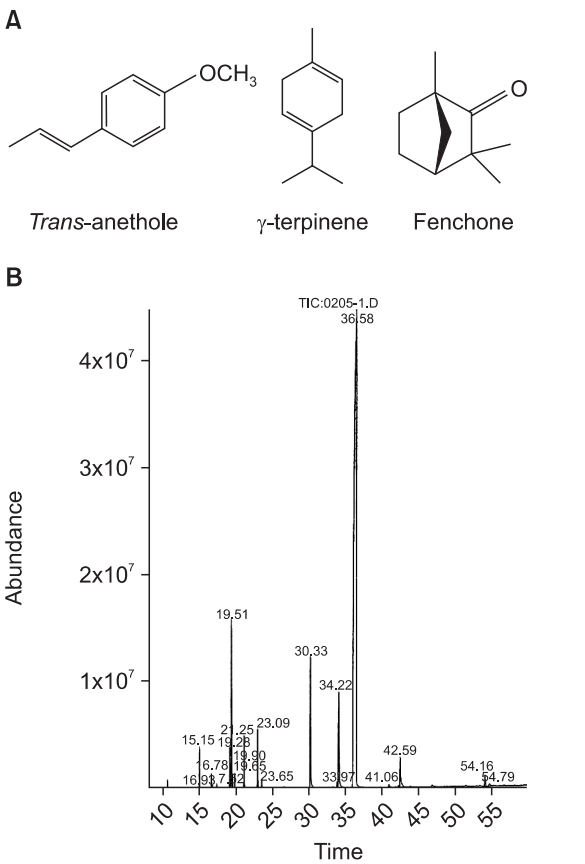


**Fig. 1.** Chemical structures of the active constituents of *F. vulgare*. (A) Chemical structures of the selected compounds isolated, (B) GC spectrum of the hexane fraction, The retention times were 15.15 (α-pinene), 16.78 (sabinene), 16.93 (β-pinene), 17.52 (myrcene), 19.28 (*p*-cymene), 19.51 (limonene), 19.65 (1,8-cineole), 19.95 (*E*-ocimene), 21.25 (γ-terpinene), 23.09 (fenchone), 30.33 (estragole), 34.22 (*p*-anisaldehyde), 36.58 (*trans*-anethole) and 42.59 min (anisylacetone).

the previously described procedure (Choi *et al.*, 1996), anti-DNP mouse IgE was added for sensitization and incubated overnight. Twenty four hours later, the cells were washed with siraganian buffer (pH 7.2). DNP-BSA (1 μg/ml) was added for activation and degranulation. The test compounds were simultaneously added. After 10 min incubation, the reaction was stopped by cooling in an ice bath for 10 min. After centrifugation, the supernatant was transferred into 96-well plates. The substrate (1 mM *p*-nitrophenyl-N-acetyl-β-D-glucosaminide) was added and incubated for 1 h at 37°C. The reaction was stopped by adding 0.1 M $Na_2CO_3$/$NaHCO_3$ (200 μl/well) and the absorbance was measured at 405 nm.

### Arachidonic acid (AA)-induced ear edema in mice and measurement of LT concentration

For establishment of in vivo inhibitory activity against 5-LOX-mediated response, AA-induced ear edema assay were carried out according to the previously reported procedures (Kim *et al.*, 1993). AA (2%) dissolved in acetone (25 μl/ear) was applied topically to mouse ear. One hour later, the ear thickness was measured using engineering gauge (Mitutoyo, Japan). Test compounds dissolved in DMSO (50 μl/mouse) were orally administered at 1 h prior to AA application.

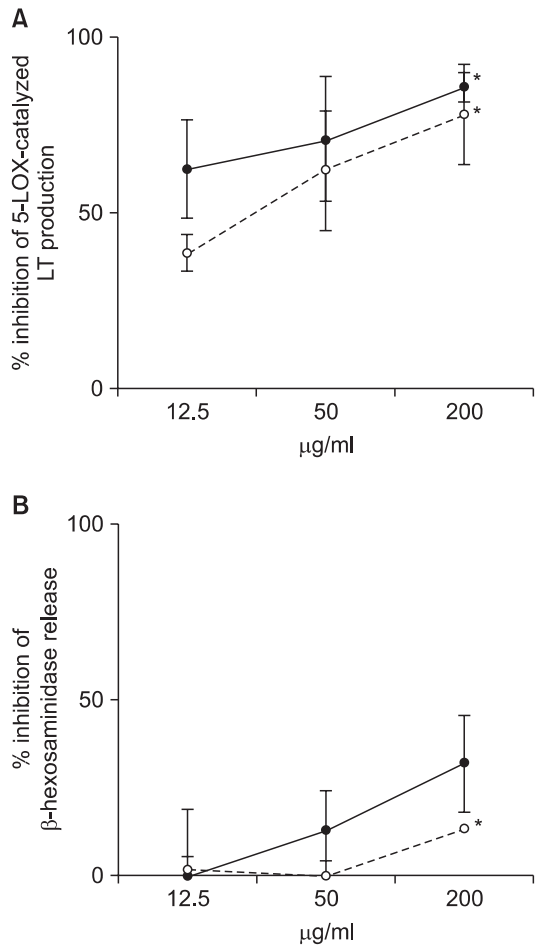


**Fig. 2.** Effects of the extracts of *F. vulgare* on 5-LOX and degranulation reaction. (A) Inhibition of 5-LOX catalyzed LT production from A23187-treated RBL-1 cells. (B) Inhibition of β-hexosaminidase release from antigen-treated RBL-2H3 cells. The water extract (○), 70% ethanol extract (●). All points and bars represent arithmetic mean ± SD (*n*=3), **p*<0.01, significantly different from the control group.

### Statistical analysis

All data were represented as arithmetic mean ± SD. One-way analysis of variance (ANOVA), followed by Dunnett's test was used to determine the statistical significance.

## RESULTS

A-23187 (ionophore) treatment to RBL-1 cells activates 5-LOX, which produces high concentrations of cysteinyl-LTs. A-23187 treatment increased LT concentrations to 749.5 ± 294.0 pg/ml from a basal level of 44.9 ± 17.5 pg (*n*=3). The water and 70% ethanol extracts of the fruits of *F. vulgare* inhibited LT production under these conditions (Fig. 2A). Comparing the $IC_{50}$ values, the ethanol extract of the fruits of *F. vulgare* (FR) possessed a higher inhibitory activity (3.2 μg/ml) against 5-LOX on activated RBL-1 than that of the water extract (25.4 μg /ml). The reference compound, NDGA, showed 92% inhibition at 1 μM.

By antigenic stimulation, mast cells release histamine which produces vasodilation and itching. Along with histamine production, β-hexosaminidase is also released. Thus, β-hexosaminidase release could be used as a biomarker in RBL-2H3 cells. When the anti-allergenic activities of FR and the water extract were evaluated, both extracts, however, showed weak inhibitory activity on degranulation of RBL-2H3 cells. The

**Table 1.** Inhibition of the constituents of the fruits of *F. vulgare* against 5-LOX-catalyzed LT production

| Compounds | % inhibition at 50 μM[a] |
|---|---|
| | 5-LOX |
| NDGA | 92.4 |
| Monoterpenes | |
| Sabinene | 22.3 |
| Fenchone | 40.9 (>50)[b] |
| γ-Terpinene | 48.3 (>50) |
| α-Pinene | –[c] |
| Limonene | 9.9 |
| Phenylpropanoids | |
| Anisylacetone | 32.6 (>50) |
| 4-Allylanisole (estragole) | – |
| *Trans*-anethole | 49.1 (51.6) |
| Phenolic | |
| *p*-Anisaldehyde | – |
| Coumarins | |
| Scopoletin | – |
| Bergapten | – |
| Umbelliferone | – |

[a]All values are arithmetic mean of % inhibition at 50 μM except NDGA (1 μM). *n*=3, [b]The values of the parenthesis are $IC_{50}$ vales in μM. [c]–: not active.

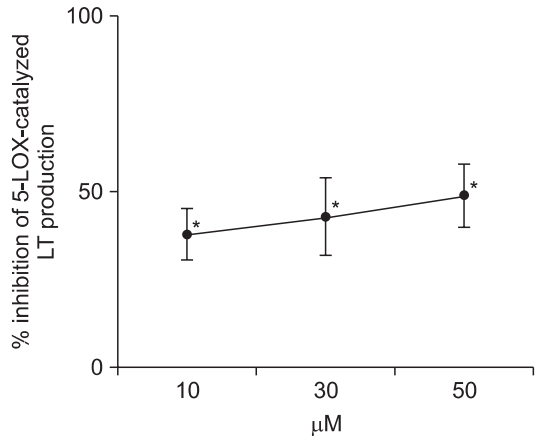


**Fig. 3.** Inhibition of *trans*-anethole on 5-LOX-catalyzed LT production. $LTC_4/D_4/E_4$ (1,624.8 ± 59.3 pg/ml) was produced from A23187-treated RBL-1 cells for 15 min incubation. Basal level of $LTC_4/D_4/E_4$ was 286.0 ± 29.0 pg/ml. All points and bars represent arithmetic mean ± SD (*n*=3), **p*<0.01, significantly different from the control group.

ethanol extract (FR) showed 32.6% inhibition while the water extract only showed 13.8% inhibition at 200 μg/ml, (Fig. 2B). Notably, FR and the water extract showed some cytotoxicity as determined by an MTT assay at this concentration (data not shown). The reference compound, quercetin, showed 80.0% inhibition at 10 μM.

Twelve major constituents were successfully isolated from *F. vulgare*. They include monoterpenes such as sabinene, fenchone, γ-terpinene, α-pinene and limonene, phenylpropanoids such as estragole (4-allylanisole) and *trans*-anethole, aromatic compounds such as *p*-anisylaldehyde and *p*-anisylacetone, and coumarins such as scopoletin, bergapten and umbelliferone. When the inhibitory activities of these constituents were examined at 2, 10 and 50 μM to determine the active principle (s), several compounds including *p*-anisylacetone, *trans*-anethole, fenchon and γ-terpinene showed more than a 30% inhibition at 50 μM against 5-LOX-catalyzed LT production in RBL-1 cells (Table 1). Particularly, *trans*-anethole showed strong inhibitory action, with an established $IC_{50}$ of 51.6 μM (Fig. 3). In contrast, all isolated constituents including *trans*-anethole did not show considerable inhibition against the degranulation reaction in RBL-2H3 cells (data not shown).

Based on these results and its high content in the extract, *trans*-anethole was selected for further in vivo study. In an AA-induced ear edema assay, ear thickness increased to 0.292 ± 0.016 mm from a control level of 0.212 ± 0.008 mm after topical treatment of 2% AA (*n*=5). NDGA used as reference (LOX inhibitor) showed significant inhibition by topical application. When administered orally to mice, FR and *trans*-anethole significantly and potently inhibited AA-induced ear edema (Table 2).

**Table 2.** Inhibition of arachidonic acid (AA)-induced ear edema in mice by FR and trans-anethole

| Compounds | Dose (mg/kg)[a] | Ear thickness increased (mm) | % inhibition |
|---|---|---|---|
| AA-treated | − | 0.080 ± 0.016 | − |
| NDGA | 2[b] | 0.044 ± 0.013* | 45.0 |
| FR | 100[c] | 0.044 ± 0.013* | 45.0 |
| *Trans*-anethole | 50 | 0.038 ± 0.017* | 52.5 |
| | 200 | 0.036 ± 0.015* | 72.5 |

[a]All compounds were orally treated one hour prior to AA application, except NDGA. [b]NDGA (2 mg/ear) was treated topically to ears of mice 30 min prior to AA application. [c]Higher dose than 100 mg/kg could not be tested due to the insolubility of the extract in DMSO. All data are arithmetic means ± SD (*n*=5). **p*<0.01, significantly different from the AA-treated group.

## DISCUSSION

The present investigation demonstrated that FR and some of its constituents possess 5-LOX inhibitory activity. It is also suggested that *trans*-anethole may contribute, at least in part, to the pharmacological activity of FR. Our study is significant since in vivo activity of FR and *trans*-anethole was demonstrated and several constituents such *trans*-anethole and γ-terpinene were found to be the active components in FR as 5-LOX inhibitors, for the first time.

Some constituents of the fruits of *F. vulgare* were previously reported to possess several pharmacological activities. For example, T-lymphocyte proliferation and IL-2 production were inhibited by anethole (Yea *et al.*, 2006). Anethole also showed a preventive effect against thrombosis (Tognolini *et al.*, 2007). Recently, anethole and limonene inhibited NO production from RAW 264.7 cells (Conforti *et al.*, 2010). In our recent study, monoterpenes such as pinene, cineole and limonene did not considerably inhibit 5-LOX from mast cells (Jin *et al.*, 2011). On the other hand, the present study demonstrated that several monoterpene derivatives such as fenchone and γ-terpinene are 5-LOX inhibitors.

AA topically applied to the ears of mice produces acute inflammation characterized by inflammatory cell recruitment and edema, peaking at 1 h (Inoue *et al.*, 1988; Kim *et al.*, 1993). In this model, AA topically applied to mice ear is converted to LTs via 5-LOX, which evokes edema in 1 h. Thus, this model is sensitive to 5-LOX inhibitors. Indeed, NDGA (5-LOX inhibitor) used as a reference drug showed significant inhibition in this model. Therefore, it is reasonably suggested that FR and *trans*-anethole might inhibit 5-LOX in ears of mice, leading to the reduction of edema in vivo.

In conclusion, FR and several of its constituents such as *trans*-anethole, fenchone and γ-terpinene were found to be 5-LOX inhibitors. In particular, FR and *trans*-anethole showed *in vivo* inhibitory activity against AA-induced ear edema in mice, possibly via 5-LOX inhibition. These results suggest that FR and *trans*-anethole may be beneficial for treating 5-LOX-related disorders and *trans*-anethole may certainly contribute to the pharmacological action of FR.

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